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14. ABSTRACT

Purpose: Develop an immunohistochemical method for identifying stem cells and stem cell niches, and to use this to determine if in utero estrogenic over-stimulation causes changes in the number of stem cells or their niches. Scope: To extend the power of ex vivo stem cell isolation and enumeration by providing a way to identify functional cell types in situ. This identification method should ultimately provide a diagnostic refinement for mammary cancers. Findings: We had marginal success due primarily to 1) most antibodies previously reputed to be "stem cell specific" turned out to be present in differentiated mammary cell types as well as putative progenitor cells; 2) some of these antibodies stained all cell types but not all members of each subset; 3) technical limitations: our inability to detect 2 to 3 antibodies simultaneously with hematoxylin counterstain. Due to these events, as with ex vivo characterization, we were unable to discover definitive markers for any cell type, however we did show that P63, originally thought to be a stem cell marker, but shown later to be an epithelial stratification organizer, is expressed differentially in the basal and luminal cells depending on the stage of estrus cycle, and whether the tissue is tumorigenic. Due to the technical challenges the main animal study was not realized.

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Stem cells, stem cell niche, immunohistochemistry, mammary gland, breast cancer

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Table of Contents

Introduction……....4

Body……4

Key Research Accomplishments……8

Reportable Outcomes……8

Conclusions……..9

References……14

Appendices……None

Introduction:

Subject: Since some mammary cancers potentially originate in stem or progenitor cells, it is important to confirm this in animal models and develop tools for investigating the process. We had already developed an in situ tool for morphologically identifying putative stem and progenitor cells in mammary epithelium at the light level (Chepko et al., 2005) which we published the year after the we accepted the BC033336. For BC033336 we proposed to use antibodies shown at that time to be effective in FACS and some *in situ* studies for differentiating stem/progenitor cell populations from differentiated cells to molecularly define and map the stem niche cells *in situ*. Secondly, because *in utero* estrogen treatment increases the risk of breast cancer, and steroid positive cells are known to locate in close proximity to receptor-negative proliferating cells (Clarke et al., 1997; Clarke et al., 2000), we proposed to 1) use steroid receptor and mammogenic hormone receptor antibodies to mark the putative niche cells to aid in determining if high estrogen increases the number of stem/progenitor cells in mammary epithelium of mice treated during the last third of their gestation. **Purpose**: To address the limitations of *ex vivo* molecular stem cell characterization and create consensus between histological and FACS data on stem cells. Limitations include the inability to 1) define a stem cell; and 2) locate it *in vivo* for purposes of determining its interactions with non-stem epithelial cells, and how the effects of hormones and growth factors are communicated to the stem cell population *in situ*; 3) *in situ* enumeration and mapping for the purpose of understanding maintenance of tissue architecture.
Scope: To develop *in situ* tools that will complement information gained from cell isolation (FACS) studies and can be used to study *in situ* preneoplastic processes, and help to demonstrate the role of stem and/or progenitor cells in cancer.

Body

Task I (Month 1-3): Experiment set-up.

A. Obtain 24 female FVB mice and inject 8 mice with peanut oil vehicle, 8 with 0.1µg 17 beta-estradiol in peanut oil, and 8 mice with 1.0µg 17-beta-estradiol in peanut oil from gestation days 14-20.
B. Sacrifice 10 mice/group at 21 days age, (10mice/group) at 50 days and (10 mice/group) at 150 days.
C. Fix the mammary glands with a solution of 2.5% paraformaldehyde and 1.25% glutaraldehyde in 0.05M PBS and embed in paraffin.
D. Section entire gland serially at 4µm.

Results: Preliminary staining of archival FVB mammary epithelium using Oct-4, Bmi-1 and Sca-1 was undertaken while awaiting approval of the grant BCO33336. These early results informed us that developing staining protocols for the antibodies used in FACS studies was not only time consuming, but was also producing unexpected results. For instance, instead of staining only rare cells, as expected, or even cells of only one epithelial class, Oct-4 stained about 50 % of all cell types, a result opposite to those reported for other stem cells at the time. When the grant was approved we decided to move forward to task II with the protocol troubleshooting using tissue blocks left over from earlier studies to 1) avoid using up experimental tissues just for developing staining protocols, and 2) allow for a case in which the results may indicate a need for experiment re-design. Although we originally proposed to use 2.5% paraformaldehyde/ 1.25% glutaraldehyde to attain a further improved structure and the lowest number of methylene cross-links, we employed the unused control tissue (fixed for 6 hours in 4% paraformaldehyde) from a study we had just

finished to pursue Task II. This protocol was designed to adequately fix and still preserve the morphological detail of the putative stem and progenitor cells of the mammary epithelium. This protocol had already been established by a study that was ending at the time that BC033336 submitted for review (Chepko et al., 2005). It was used to a) preserve antigenicity and b) avoid destruction of the delicate morphological characteristics that identifying the putative stem and progenitor cells relies upon (Chepko and Smith, 1997; Chepko et al., 2005).

Task II (Month 3-4): Determine if established cell morphologies correspond with cell markers. Select markers to use for analysis.

A. Stain selected slides immunohistochemically for Oct-4, Bmi-1, P63, Flt3, Sca-1, BCRP, decapentaplagic, delta, and Notch-1.
B. Examine slides for cell morphology -- cell marker match.
C. Choose stains to use based on positive reaction.

A. Protocol Development and Staining

Materials and Methods.

Paraffin sections were cut at 4µm from normal FVB mouse mammary gland of the following developmental stages and used for immunostaining: 35 day, 5 week, 3 month-old female mice; mammary glands from 6, 10, and 17-day pregnant, and 2-day involution. Control tissues were intestine, ovary, epidermis, liver and hair follicle. Mouse mammary tumors from MMTV-c-myc and bi-transgenic MMTV c-myc-MT-TGFα over-expressing were also stained in some instances. All tissue was fixed at 4°C for 6 hours in 4% paraformaldehyde in 0.5M PBS at pH 7.2.

Immunostaining was performed at antibody dilutions ranging from 1:10 to 1:300 at 4°C overnight or at room temp for 30-60 minutes. These tests were performed using the Vectastain Elite, DAKO ARK, CSA, Envision, or Envision Plus and Envision Plus Doublestain Kits and the following antibodies: Sca-1 MAB 1226 (IgG1)(R&D Systems); ABCG2 (BCRP) MAB 995 (IgG2b) (R&D Systems); Oct-4 sc-5279 (IgG2B) (Santa Cruz); P63 ab3239 (IgG2a) (Abcam); Notch-1 ab1 MS-1339-P1 (IgG2b) (NeoMarkers); PR-AT 4:14 anti-Progesterone receptor (PR) against peptides 533-547 of human progesterone receptor (a gift of Dr. Abdulmaged Traish); anti-estrogen receptor (ER) 78-1 (also a gift of Dr. Traish); CD44 Clone DF1485 (IgG1) Zymed Laboratories; CD49f Cat #RDI-M1566clb, Clone #NKI-GoH3 (Fitzgerald Industries International); Bmi-1, clone 229F6 (IgG1) Cat # 05-637 (Upstate Biotechnology); anti-Prolactin Receptor (Prl-R) B6.2, NIH. All antibodies used were monoclonal.

We chose to add anti-CD44 to the list of tested antibodies because at the ultrastructural level, putative mammary stem cells are frequently enveloped by a space that represents material extracted during tissue preparation (Chepko and Smith, 1997; Chepko and Dickson, 2003). We reasoned that in life it was most likely filled with hyaluronic acid (Chepko and Dickson, 2003) which could indicate the presence of its receptor, CD44, and is known to be important in motility and present on breast cancer stem cells (Al Hajj et al., 2003).

B. Matching cell morphology to putative function

As has since been shown by FACS for all other “stem cell markers” (Shackleton et al., 2006b; Stingl et al., 2006) (Clayton et al., 2004) (Kiel and Morrison, 2006), no single stain showed any kind of cell specificity and some, such as P63, shifted cell compartment localization from nucleus to cytoplasm with developmental or physiological stage and neoplastic condition. Therefore our goal of developing a method for identifying tissue specific stem cells *in situ* still eludes us.

Results:

Our attempts to use more than one stain to simultaneously differentiate two markers in the same section were discarded after discovering that the second stain (Fast Red) was not easily differentiated from the brown DAB, it drifted from its original place in the tissue over time, and required a mountant that compromised our ability to discern the different cell morphologies that were to be used to match structure and function.

Notch-1. Notch proteins are known to act in cell fate determination in many tissues and are associated with stem cell niche function in Drosophila ovary (Xie and Spradling, 2000), and in mammalian cancers (Weng and Aster, 2004). Staining was sporadic throughout the epithelium, but when present many cells in the structure (ducts or lobular epithelium) stained, not just a few or the putative stem, progenitor or niche cells. It was absent in larger ducts (Fig.1A&B).

Oct-4 showed nuclear localization in normal mammary and epidermal epithelia, and cytoplasmic localization in tumors (Fig. Fig. 2 C&D). This result was unexpected since many embryonic and hematopoietic stem cell investigators reported at the time (Mitalipov et al., 2003; Parfenov et al., 2003; Pochampally et al., 2004; Prusa et al., 2003), that Oct-4 is expressed solely by the first 2-4 cells of the early embryo and later by some cells in the blastocyst.

A subset of all cell types in mammary epithelium was positive for Oct-4, and roughly 50% of all mammary epithelial cells were Oct-4 positive (Fig. 2 C). This was also true for epidermis and hair follicle (Fig. 2 B&D). Oct-4 was present in c-myc-TGFα mammary tumors (Fig 2A). Stem cells are believed to be a rare cell type in all tissues, therefore because so many mammary and epidermal cells are positive for Oct-4, it cannot be used to demonstrate stem cell localization *in situ*. In epidermis sebaceous cells were Oct-4 positive whilst they were negative for P63.

CD44, and CD49f were ubiquitous for epithelium and connective tissue alike (not shown), and was therefore found to be a useless in situ marker for any kind of cells.

Staining for P63 (Fig. 3) shifted randomly during pregnancy from the stroma to myoepithelial cell nuclei to luminal cell cytoplasm to capillary endothelium, showing no consistency within a single section or particular stage. At 2 days involution the mast cell cytoplasm, some myoepithelial cell nuclei, and the luminal cell cytoplasm were P63 positive. In the 5 week old mammary gland P63 stained nuclei of myoepithelial cells, cap cells and body cells in the terminal end buds and occasional putative progenitor cells in the ducts. The cytoplasm of the capillary endothelial cells, some of the body cells of the terminal end buds, and the fat cells was positive. In the 3 month glands of nulliparous animals both luminal epithelium and myoepithelium may or may not be positive, with some parts of the gland having P63 positive cells and other parts being entirely negative. Ten day pregnant animals showed the same sporadic staining pattern (Fig 3 D&E). The stain could be either in the cytoplasm or the nuclei, and the pattern was complex enough to appear to be random. There are two main isotypes of P63 which mediate different functions during the process of stratification in the epidermis. TA isoforms contain a amino transactivation domain and those lacking this domain, but that are also capable of gene expression transactivation (Koster et al., 2004). Koster et al have shown that P63 initiates epithelial stratification during development and maintains proliferative potential after maturity. The antibody we used recognizes all forms of P63 and since the mammary gland is under a regimen of continual self renewal and tissue modeling it may play these roles alternately throughout reproductive life in this organ. Evidence that points to this is the changing pattern of stain from the basal myoepithelium to the luminal epithelium. The sporadic staining pattern may be due to the fact that growth and remodeling in the mammary epithelium is not uniform throughout the gland at any given time, although it is generally coordinated by the estrus cycle. P63 positive cells were rare in c-myc and c-myc/TGFα bi-transgenic mammary tumors.

Bmi-1 (Fig. 4) showed sporadic staining for mammary epithelium within specificity for cell type or epithelial structure, and stained numerous cells positively while other whole structures remained negative within the same section. Again, this Pou domain protein did not demonstrate the specificity necessary to define rare cells.

Sca-1 and *ABCG2 (Bcrp-1)* were expressed at such low levels they demonstrated no immunostaining with the available antibodies.

The protocol development took the rest of the year and the following year. We attempted to increase the staining signal, and stain for more than one marker at a time (as is done by FACS) by trying Qdot nanocrystals (Invitrogen), with some success with P63. However, we lacked the proper filter set which would allow simultaneous visualization of all the markers used.

C. Choose stains to use based on positive reaction.

By mid-2006 none of the stains we used had demonstrated any cellular specificity, and because by 2006 Shakleton et al and Stingl et al (Shackleton et al., 2006a; Stingl et al., 2006) had demonstrated in mammary epithelium that no marker used alone was able to define a stem cell or any other kind of mammary epithelial cell we were unable to carry out this portion of task II.

Task III (Month 4-12): Marker analysis, cell counting and data analysis.

A. Stain serial sections according to decision above. Randomize slides and fields to be counted.
B. Capture electronic images in serial sections.
C. Count cells in Image-Pro based on cell type and/or stem/progenitor cell marker.
D. Molecularly characterize the populations of normal mammary stem, and progenitor cells and the stem cell niche. Compare with estrogen treated data.
E. Stack images and perform appropriate 3-dimensional statistical analysis on niche number, and spacing, cell type ratios and cell-cell relationships with help of Lombardi Comprehensive Cancer Center Imaging and Biostatics Cores.
F. Analyze data to determine 1) the normal ratio of stem and progenitor cells in treated versus untreated mammary epithelium, 2) the normal spacing of niches, 3) whether an elevated *in utero* estrogenic environment affects the ratio(s) of these populations and the niche spacing.

Although we captured 1.06 gigabytes of electronic images from our staining of approximately 500 stained sections, our results gave no reason to proceed with sections A & C through F of Task III. However, included in the sections were those from 35 day mice which had been implanted with progesterone, estrogen or cholesterol vehicle (control) pellets at the nape of their necks at 25 days of age (tissue from archival blocks from previous studies). We decided to use these for estrogen (ER), progesterone (PR) and prolactin receptor (PrlR) staining in lieu of the *in utero* estrogen injection study (Task I) since the results of the protocol development had informed against pursing the original experimental design. These stains were somewhat more informative because the PR stain produced some of the expected pattern. All three antibodies often stained the putative progenitor cells and a subset of the putative stem cells (Fig. 5), whilst the niche-like cells were negative. However, subsets of all cell types except myoepithelial cells and niche-like cells were positive for PR and ER. Myoepithelial cells were uniformly negative for PR and PrlR. PrlR stained the putative progenitors.

Accomplishments and Future Directions: Work on stem cells in mammary gland is moving steadily toward the establishment of a mammary epithelial stem cell hierarchy (Paguirigan et al., 2007) and the signaling network that governs it (Ewan et al., 2005; Barcellos-Hoff and Ravani, 2000; Ewan et al., 2005). Better

programs for enumerating cell types *in situ* are coming into availability, and so are better ways to model stem cell hierarchies in solid tissues (Paguirigan and Beebe, 2006). It is clear that studying stem cells in situ, which we must if we are to really understand the relationship of development to tumorigenesis and the signaling networks that govern the two processes, we will need to incorporate these new tools into our future inquiries. The other exciting new technology that was developed while we were involved with this study is Qdot labled antibodies (Invitrogen) and the filter for visualizing up to 8 at a time *in situ*. This may have helped to eliminate the noise and allow us to focus on the proper combination of negative and positive signaling as well as definitive morphology.

Key research accomplishments:

- P63 is neither a stem cell marker nor limited to myoepithelial cell nuclei (as we believed at the start of this work). Instead, in the mammary gland it changes location from myoepithelial nuclei to the nuclei and cytoplasm of the luminal cells. In epidermis it was shown to be important in generating and maintaining stratification. Since the mammary epithelium is also stratified (between contractile myoepithelial and luminal secretory cells) it may play a similar role in mammary gland. If this is so, it may be essential in tumor suppression. Antibodies against the specific isoforms may be helpful in determining this.
- Notch-1 stains many different cell types. It may still be useful in combination with other markers once new technologies are explored.
- The hormone receptor signaling gave us a staining pattern similar to our expectations for the stem cell niche and progenitor cells. Perhaps combination staining using Qdots will be useful in defining the action of hormones on stem cell hierarchies and their members.
- Oct-4 is ubiquitous in mammary gland and epidermis. This is a surprising result as it has recently been reported to be a tumor cell marker (Baker and Oliva, 2005; Howell et al., 2007). However, if it is so common in normal epithelia, it cannot be either a tumor maker or a stem cell biomarker. However, Oct-4 could be important in epigenetic regulation, since as a POU factor it is involved in gene repression.
- There are presently no good antibodies to either BCRP or Sca-1 for use in paraffin sections. However, between 2003 and 2005 it became clear that contrary to general belief at the time this grant was written, neither of these (nor any other marker) is sufficient for defining stem or progenitor cells. Strangely, for *in situ* work or anything beyond a functional definition, our best definitions for mammary epithelial stem and progenitor cells remain the ultrastructural features identified in 1997 by Chepko and Smith (Chepko and Smith, 1997). It's been ten years for all the groups working on it, so it's a hard job. We need to keep at it using newly developed technology and applying it to *in situ* visualization and enumeration through dynamic mammary gland morphogenesis and tumorigenesis.

Reportable Outcomes.

- Although much of this may be useful in designing and carrying out future studies, none of it is publishable or useful in more than posters or public talks.
- Some of this data was presented in a poster at the June 2005 DoD meeting in Philadelphia.

Conclusions: The main conclusion we can draw from this effort is that in order for the outcome to be meaningful a technology that offers a way to individually label multiple markers simultaneously is indispensable. When we began we thought we could make some headway with two: DAB and Fast Red, but Fast Red proved to be useless for such exacting work. Also knowledge about stem cell markers when we started was in its infancy and changed drastically by 2006, especially as it became clear that no stem cell appeared to be defined by a single marker or by its inclusion in a particular population (Stingl et al., 2006).

So What: All of the information gained is new and useful in future studies, it is of such an incidental nature because of the lack of multiple marker technology that it is not publishable in its present state. The problem is still unsolved: we still need to know how the mammary epithelium builds itself from a few stem cells, maintains its stem, progenitor, secretory, and myoepithelial cells, and resists tumorigenesis under a constant barrage of proliferation and differentiation signals. The secret to this coordination of influences is the way toward the solution of tumorigenic processes.

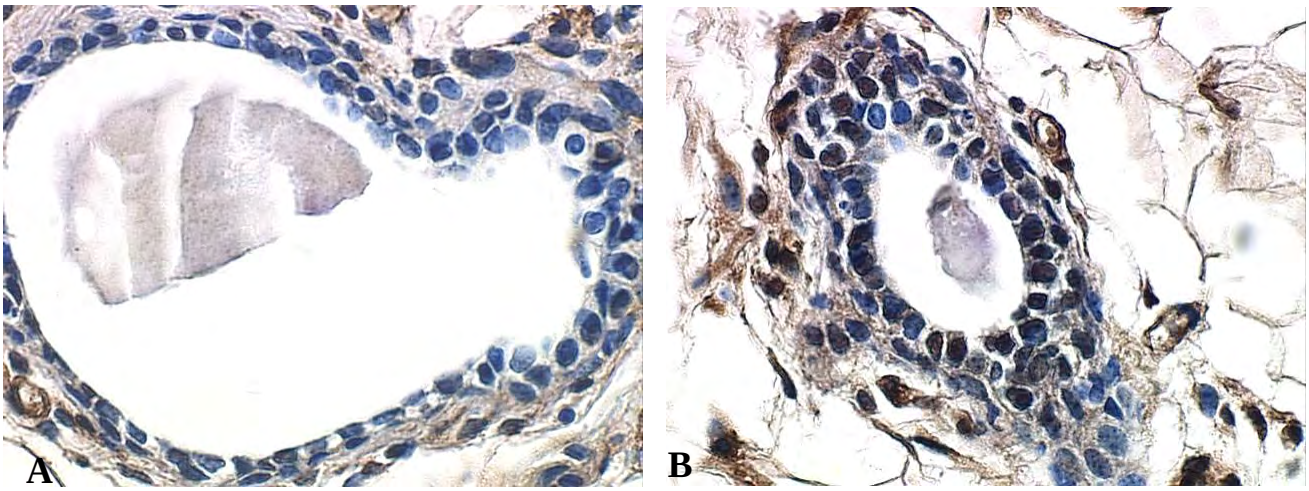

Figure1. Anti- Notch-1 staining. **A.** Large duct epithelium showing negative staining for Notch-1. **B**. Small duct epithelium with numerous positive epithelial cells.

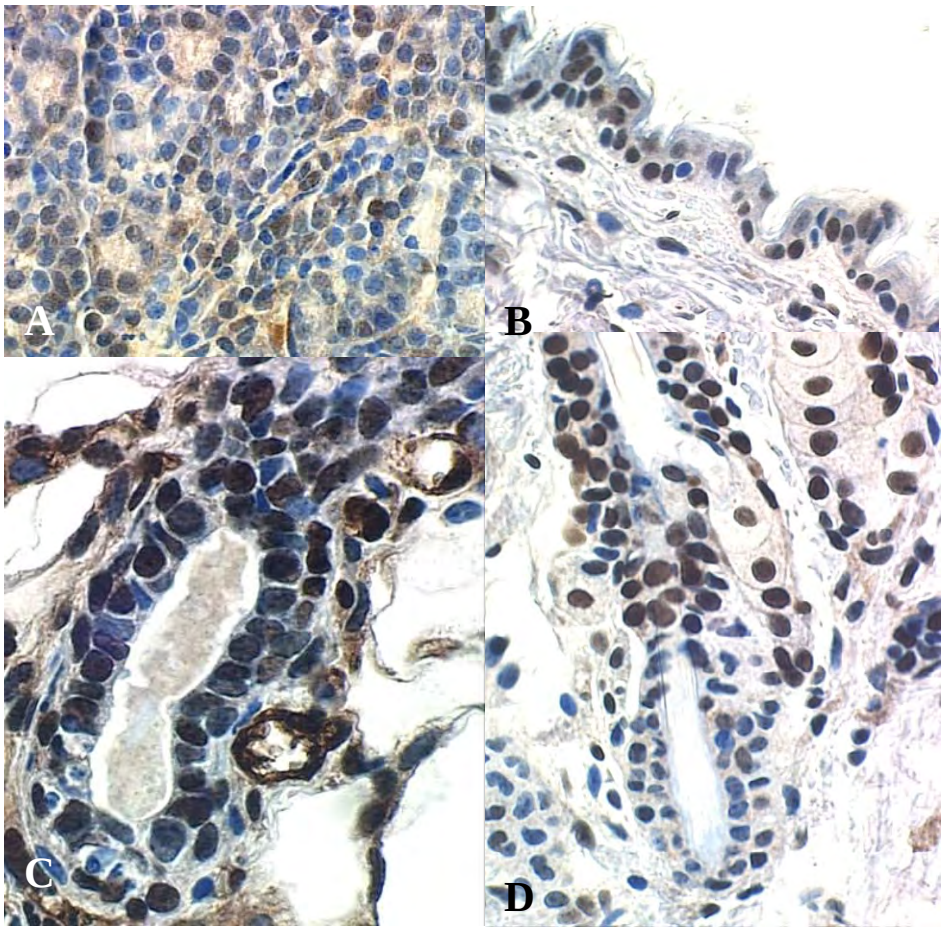

Figure 2. Oct4. P63 staining in (**A**)c-myc-TGFα bitransgenic tumor (**C**) normal adult nulliparous mammary epithelium, **(B)** epidermis and **(D)** hair follicle. Oct 4 stains at least 50% of the cells in both epithelia and since stem cells are rare it cannot serve as a stem cell marker.

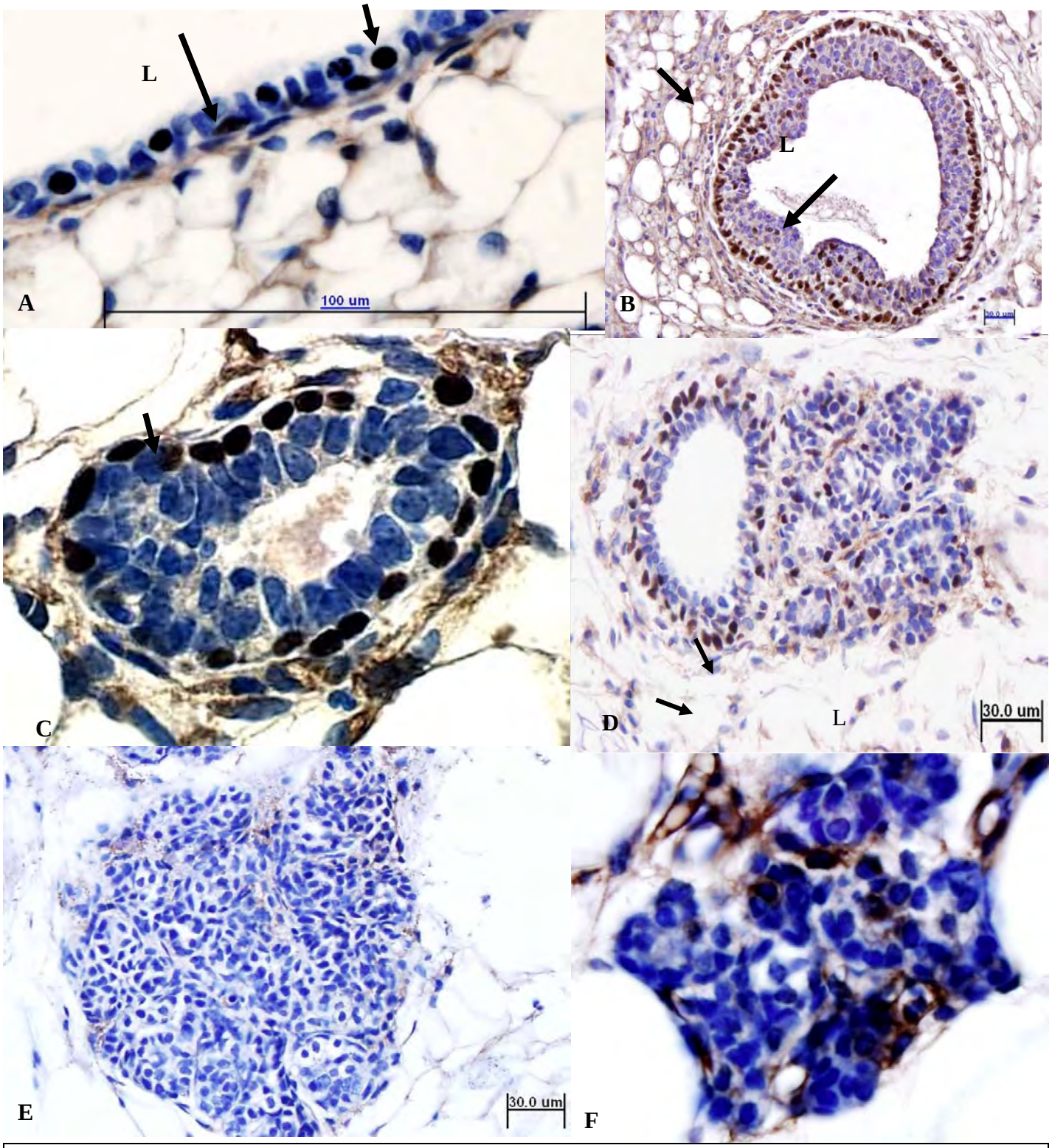

Figure 3. P63 in mouse mammary gland: **A**. Age: Five weeks; duct. Arrows: P63 positive (deep brown) putative progenitor (transit amplifying cells) **B.** Age: Five weeks; terminal end bud. Arrows: P63 positive cap or body cells L, TEB lumen. Other brown stained cells are TEB body cells. **C.** Age: 3 months; duct, cross section, myoepithelial cells are positive. **D&E.** Age: 4 months; 10 days pregnancy. **F.** 4 months; 17 days pregnant. Mammary epithelium is negative; some endothelium is P63 positive.

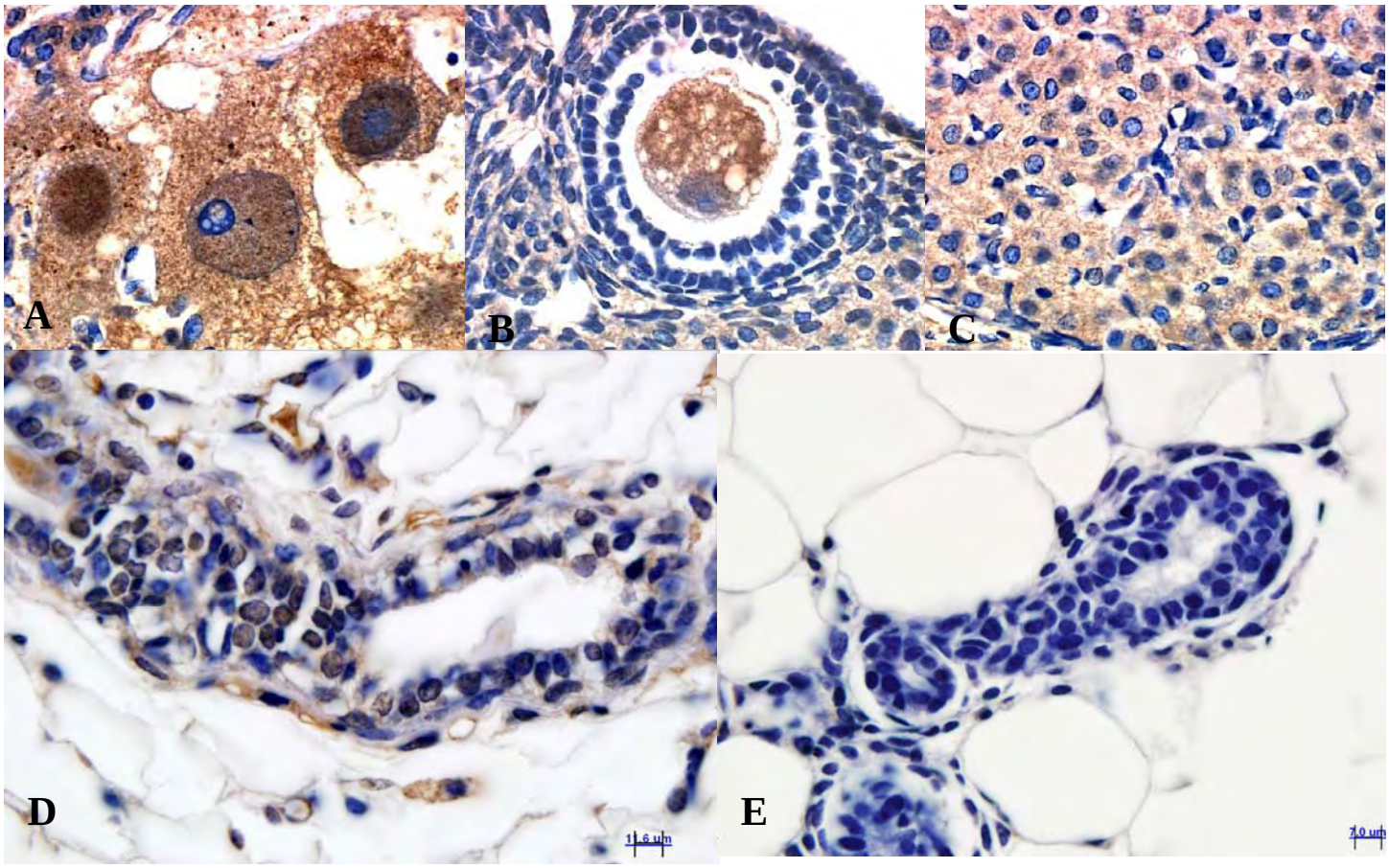

Figure 4. Bmi-1 Stain. A, B, & C: Ovary - positive tissue control. A. Oocytes with nuclear and cytoplasmic stain. B. Primary follicle with positively stained oocyte surrounded by negatively stained granulosa cells. C. Luteinizing follicular tissue – all granulocytes has positive cytoplasm. D. Mammary ductule with numerous cells with positive nuclei. E. Mammary ductule with all Bmi-1 negative cells.

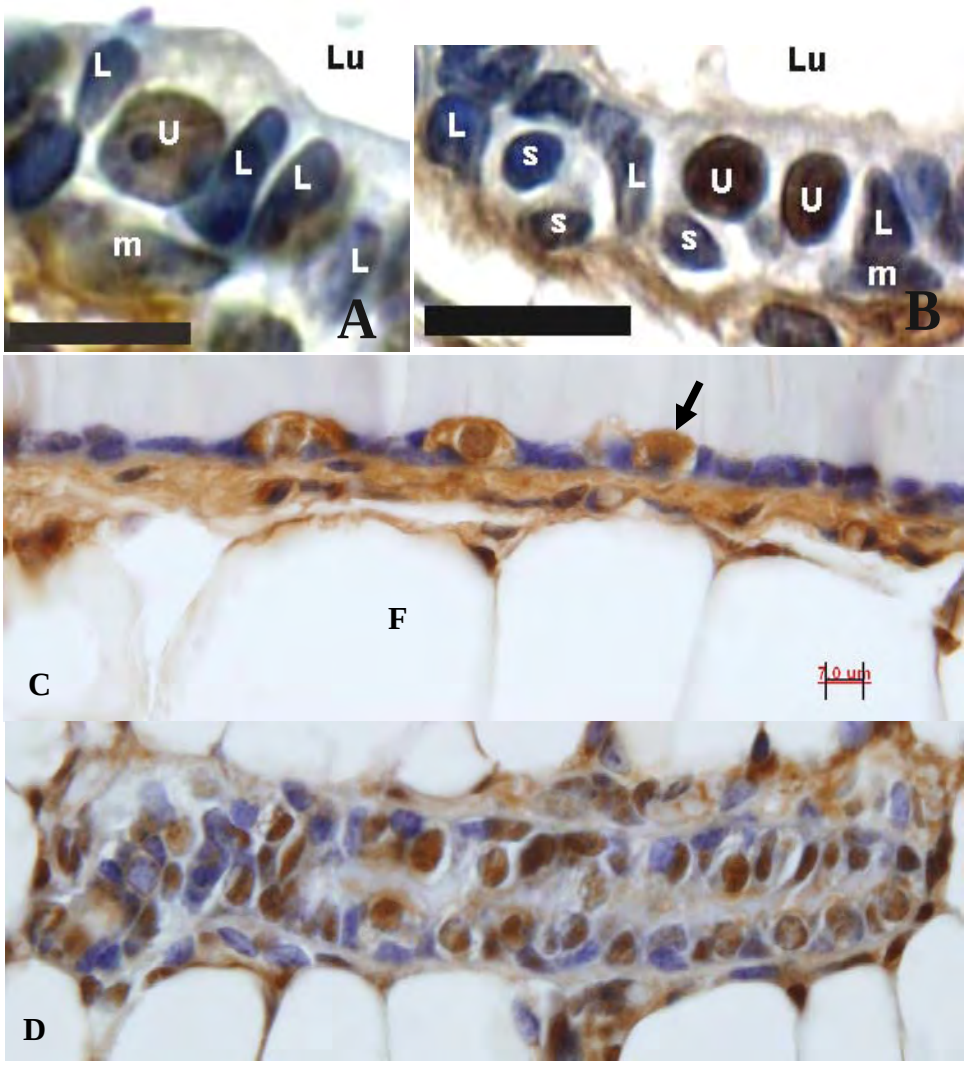

Figure 5. **A&B**: Progesterone receptor in mammary ductal epithelium of nulliparous 35 day old mouse stimulated with progesterone for 9 days. Anti-progesterone receptor (brown) is present in putative progenitor cells (U) and a subset of putative stem cells (s) but absent in niche-like cells (L). m; myoepithelial cell. Bar ~ 20µm. **C.** Prolactin receptor in adult nulliparous MG. Only the putative progenitor cells are positive (arrow). F = fat cell. **D.** Estrogen receptor in adult nulliparous MG. Mainly putative progenitors (large round nuclei), but also some putative niche cells (thin, elongate vertically oriented nuclei) and nuclei of occasional myoepithelial cells (horizontally oblong nuclei) are positive for ER. Bar for C applies to D.

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